Stomach curvature is generated by left-right asymmetric gut morphogenesis

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ABSTRACT

Left-right (LR) asymmetry is a fundamental feature of internal anatomy, yet the emergence of morphological asymmetry remains one of the least understood phases of organogenesis. Asymmetric rotation of the intestine is directed by forces outside the gut, but the morphogenetic events that generate anatomical asymmetry in other regions of the digestive tract remain unknown. Here, we show in mouse and Xenopus that the mechanisms that drive the curvature of the stomach are intrinsic to the gut tube itself. The left wall of the primitive stomach expands more than the right wall, as the left epithelium becomes more polarized and undergoes radial rearrangement. These asymmetries exist across several species, and are dependent on LR patterning genes, including Foxj1, Nodal and Pitx2. Our findings have implications for how LR patterning manifests distinct types of morphological asymmetries in different contexts.

KEY WORDS: Left-right, Asymmetry, Stomach, Gut, Morphogenesis, Pitx2, Xenopus, Mouse

INTRODUCTION

The external symmetry of bilateria belies the marked left-right (LR) asymmetry of their internal organs. In vertebrates, the LR axis is established by nodal cilia, which activate expression of the TGFβ molecule Nodal exclusively on the left side of the embryo (Levin et al., 1995; Lowe et al., 1996; Murcia et al., 2000; Okada et al., 1999). Downstream of Nodal, the transcription factor Pitx2c is then expressed along the left side of developing organs as they acquire morphological asymmetry (Campione et al., 1999). These patterning events culminate in anatomical laterality, including left- or right-side organ position, LR asymmetry in the shapes/sizes of paired organs, or intrinsic morphological disparity between the sides of an individual organ (Capdevila et al., 2000).

The J-shaped curvature of the stomach is an archetype of such laterality. The exact configuration of this organ varies among species with different diets, yet its distinctive shape, which is characterized by a longer ‘greater’ curvature on the left, and shorter ‘lesser’ curvature on the right, is highly conserved and integral to normal function (Stevens and Hume, 2004). Early embryologists contended that stomach anatomy results from a developmental rotation in which the ostensibly enlarged dorsal surface turns leftward to become the greater curvature (Liebermann-Meffert, 1969) (Fig. 1A). However, some have argued that rotation is illusory and that curvature emerges via an intrinsic process, e.g. asymmetric growth (Kanagasuntheram, 1957; Liebermann-Meffert, 1969) (Fig. 1B). Remarkably, the cellular and molecular events that drive stomach curvature have never been experimentally addressed.

RESULTS AND DISCUSSION

Stomach asymmetry is independent of rotation

We compared stomach curvature formation in two animals, the mouse and the frog (Xenopus laevis). In both species, the slit-like lumen of the still-straight primitive stomach [mouse stage E10 and frog stage 34 (Nieuwkoop and Faber, 1994)] is initially aligned with the dorsal-ventral (DV) axis of the embryo (Fig. 1C,E). If the dorsal face of the organ rotates leftward during development, we would expect its DV axis to shift clockwise with respect to the embryonic midline as asymmetric morphology emerges (in posterior view, Fig. 1A). Surprisingly, we find no indication of such a shift in either species. However, in both the mouse (Fig. 1C,D) and frog (Fig. 1E-F), the left wall of the early stomach becomes longer than the right wall and begins to bend outwards, skewing the nascent lumen leftward. These LR differences are also evident in orthogonal planes of section (not shown), indicating that a general expansion of the left wall occurs concomitantly with the emergence of overt laterality. This intrinsic asymmetry is evident prior to mesogastrial remodeling; indeed, in the frog, a mesogastrium is not detectable when stomach asymmetry becomes obvious. Thus, external tissues/forces may not be required for stomach curvature. These observations are congruent with a model of asymmetric stomach morphogenesis driven by preferential expansion of the left wall (i.e. Fig. 1B).

Differential tissue architecture develops in the left and right stomach walls

To define the morphogenetic events that drive asymmetric expansion, we compared contralateral tissue architecture during stomach development (Fig. 2). The walls of the gut tube comprise an inner layer of endoderm, which will become the epithelium, and an outer layer of mesoderm, which will become visceral muscle and connective tissue. Although the relative thickness of each layer varies between mouse and frog embryos, the tissues of the left stomach wall become significantly thinner than the right wall at stages preceding and coinciding with curvature in both species (Fig. 2A,B,E,F,I,J,M,N).

In vertebrates, the transcription factor Foxj1 is a master regulator of motile ciliogenesis required for proper establishment of the LR axis (Brody et al., 2000; Chen et al., 1998; Stubbs et al., 2008; Tamakoshi et al., 2006; Walentek et al., 2012). To determine...
whether the differential morphogenesis of the stomach walls is dependent on LR patterning, we analyzed Foxj1 mutant mouse embryos. In Foxj1 heterozygotes, which have normal LR patterning (Muthusamy et al., 2014), the preferential lengthening of the left stomach wall and asymmetric lumen expansion proceed normally (Fig. 2B). However, in Foxj1 homozygous-null embryos, in which nodal cilia are defective and LR patterning and organ morphology are consequently randomized, the length of the opposing stomach walls is equivalent and the stomach appears straightened (Fig. 2C,D; Fig. S1). Moreover, there is no LR asymmetry in the thickness of the tissue layers (Fig. 2G,H).

LR asymmetry is propagated by the expression of nodal on the left side of the embryo (Levin et al., 1995; Lowe et al., 1996; Murcia et al., 2000; Okada et al., 1999). Xenopus neurulae exposed to a small molecule inhibitor of the Nodal receptor (e.g. SB505124; Fig. S2A,B) have decreased Nodal signaling, perturbed laterality and aberrant organogenesis (Dush et al., 2011). In such embryos, the lengths of the contralateral stomach walls are equal (Fig. S2C,D; Fig. S1). Moreover, there is no LR asymmetry in the thickness of the tissue layers (Fig. 2G,H).

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Fig. 1. Early stomach undergoes leftward expansion. The rotation model (A) posits that the embryonic stomach (shown in ventral views and cross-sections at successive stages) rotates around its longitudinal axis, shifting its dorsal face leftward. An alternative model (B) theorizes that the left wall expands more than the right. Sections of E10.5, E11 and E11.5 mouse embryos (C) or stage 34, 37 and 39 frog embryos (E) reveal the leftward expansion of the early stomach. The left/right ratio of the lengths of the stomach walls becomes significantly greater than 1 in mouse by E11 (D) and in frog by stage 39 (F); *P<0.05. Sections in C and E are false-colored to match diagrams in A and B, highlighting layers of the stomach: right mesoderm, pink; right endoderm, teal; left endoderm, blue; left mesoderm, gold. In all sections, dorsal is upwards and the left side of animal is on right side of image. D, dorsal; V, ventral; L, left; R, right. Scale bars: 500 µm in C (E11.5, 150 µm); 75 µm in E.

Stomach curvature is accompanied by left-right asymmetric endoderm rearrangement

As radial rearrangement of the endoderm thins and expands other regions of the gut tube (Chalmers and Slack, 2000; Reed et al., 2009), we compared the number of endoderm cell layers within the left and right stomach walls. Prior to curvature, there are no LR differences (L/R ratio ~1). However, as curvature emerges, fewer layers are apparent in the left wall (L/R ratio<1; average mouse=0.44, P<0.01; frog=0.77, P<0.01; Fig. 2Q-T). As the total number and shape of cells on each side remains equivalent during curvature (Fig. S3A-C), these results indicate that preferential expansion of the left wall is driven by LR asymmetric cell rearrangement.

Appearing concomitantly with LR differences in cell rearrangement are asymmetries in endoderm cell polarity. This is not surprising, as polarity is a prerequisite for cells to undergo radial rearrangement (Marsden and Desimone, 2001) and endoderm cells acquire apicobasal polarity as they rearrange to lengthen the intestine and form the mature digestive epithelium (Grosse et al., 2011; Matsumoto et al., 2002; Reed et al., 2009). In Foxj1 heterozygous mice, the endoderm of the posterior right stomach wall becomes irregularly stratified, whereas the left endoderm is highly polarized (as indicated by apical enrichment of tubulin;
This LR asymmetric tissue architecture is variably perturbed in heterotaxic Foxj1−/− embryos, consistent with the randomization of LR patterning cues (Zhang et al., 2004). In one Foxj1−/− embryo, both left and right walls of the straightened stomach are highly polarized (Fig. 3B,E,H), and the number of endoderm cell layers remains equivalent (average L/R ratio=1.19, P<0.01; Fig. 2Q,R); in another case, however, both sides exhibit irregular architecture (Fig. 3C,F,I).

In frogs, the endoderm is substantially thicker than in the mouse; nonetheless, similar LR differences appear as the left endoderm is substantially thicker than the right in frogs (Fig. 2I-L). In Foxj1+/- controls (E11.5), the lumen expands leftward and left-right differences in tissue width are evident (B); however, in Foxj1−/− mutants, the normal leftward expansion of the stomach is perturbed (C,D), and left-right differences in tissue width are eliminated (G,H). Likewise, frog embryos exposed to DMSO show normal leftward expansion of the stomach lumen (K); this is eliminated in embryos exposed to a Nodal inhibitor (SB505124; L), as are normal left-right differences in widths of endoderm and mesoderm (O,P). Nuclear staining reveals asymmetry in the number of endoderm cell layers in the right (teal) versus left (blue) stomach walls by E11.5 in mouse (Q,R) and stage 39 in frog (S,T); this asymmetry is perturbed in Foxj1−/− mutants (Q,R) and in frog embryos exposed to SB505124 (S,T). Scale bars: 100 µm in A-D,Q; 75 µm in I-L,S. *P<0.01; NS, not significant.
endoderm adopts a more polarized morphology (Fig. 3J,L,N; Fig. S4A,B,E,H). Interestingly, in the straightened stomachs of Nodal-deficient (i.e. SB505124-treated) frog embryos, the left endoderm closely resembles the normal right side (Fig. 3K,M,O) and the number of cell layers remains equivalent (average L/R ratio=1.16, \( P<0.01 \); Fig. 2S-T). Thus, in both species, the emergence of morphological stomach laterality correlates with LR asymmetries in endoderm polarity and rearrangement.

Pitx2 controls left stomach epithelial morphogenesis

The expression of Pitx2c is limited to the left wall during stomach development (Liu et al., 2001; Logan et al., 1998) and is required for normal organ laterality (Bamforth et al., 2004; Campione et al., 1999; Liu et al., 2001; Logan et al., 1998; Ryan et al., 1998). Pitx2c-null mice may exhibit reversed or midline stomach orientations (Bamforth et al., 2004), although the mechanism by which Pitx2 impacts stomach morphogenesis is unknown. In other regions of the digestive tract, Pitx isoforms are associated with polarized epithelial morphogenesis and tissue expansion (e.g. intestine lengthening, cecal budding) (Al et al., 2012; Campione et al., 1999; Chung et al., 2011). Thus, asymmetric expression of Pitx2c in left stomach may drive the observed asymmetries in endoderm morphogenesis.

Consistent with this hypothesis, CRISPR-Cas9-mediated mutation of Xenopus pitx2c results in straightened stomachs in which the left wall does not lengthen or thin, and exhibits abnormal epithelial architecture (Fig. S5) and failed cell rearrangement [as indicated by an increased number of cell layers in the left epithelium (average L/R ratio=1.31, \( P<0.05 \)], confirming that pitx2c is specifically required for key aspects of asymmetric stomach morphogenesis. However, as CRISPR-Cas9 editing introduces random mutations (Fig. S5E), it is impossible to define spatially the exact cells harboring null mutations and the extent to which Pitx2 activity is altered in each F0 animal. Therefore, to specifically determine the role of pitx2c within left stomach, we also capitalized on the amenability of Xenopus for targeting gain- or loss-of-function reagents specifically to the left or right side of developing organs by microinjection, a strategy not easily feasible in mouse (Blum et al., 2009; Duncan and Khokha, 2016).

First, we employed two independent morpholino oligonucleotides (MOs) to deplete Pitx2c translation in a targeted manner (see Fig. S6A,B). When pitx2c MO is microinjected to target the right stomach, stomach curvature is unaffected (8%; \( n=37 \)); however, if pitx2c MO is targeted to the left side, the organ fails to curve [41% reduced/absent greater curvature (\( n=37 \)], compared with control MO, 4% (\( n=41 \)], recapitulating the CRISPR-Cas9 phenotype (Fig. 4A,B;
Concomitant with lack of curvature, the Pitx2c-deficient left epithelium now resembles the less organized and unpolarized right epithelium (Fig. 4E,F,I,J,N,R; Fig. S4C,F,I). The pitx2c MO-injected left wall does not thin (Fig. S6D,E), nor do the endoderm cells rearrange, as indicated by increased cell layers on the left (Fig. S6F; average L/R ratio control MO=0.83; pitx2c MO=1.34). Interestingly, the endoderm and mesoderm layers of the stomach wall may influence each other during asymmetric morphogenesis, as MO-injected clones limited primarily to the endoderm or mesoderm layer also perturb the architecture of neighboring uninjected tissue (Fig. S7). This reciprocal influence is well established in foregut patterning (Womble et al., 2016).

To determine whether pitx2c alone is sufficient to impart apicobasal polarity to the endoderm, we targeted mRNA encoding a glucocorticoid-inducible version of Pitx2c, pitx2c-GR (Chung et al., 2011), to the right stomach. Induction of ectopic Pitx2c activity by dexamethasone eliminates stomach curvature (60%, n=82; Fig. 4C,D), while uninduced control stomachs remain unaffected (10%; n=90). Strikingly, inducing Pitx2c-GR results in ectopic polarization of the right endoderm, with the affected tissue expanding into the stomach lumen (Fig. 4G,H,K,L) and expressing higher levels of polarity markers compared with adjacent uninduced tissue (Fig. 4O,P,S,T; Fig. S4D,G,J). Thus, pitx2c is necessary and sufficient to promote epithelial polarization in stomach endoderm.

How anatomical asymmetries are generated is relevant to the origin of novelty during evolution and integral to the etiology of birth defects but, for most organs, this crucial phase of organogenesis is poorly understood. Recent work suggests asymmetrical intestinal rotation is generated by LR differences in the dorsal mesentery that connects the midgut to the body wall – not in the gut tube itself, which remains symmetrical during the rotation process (Davis et al., 2008; Kurpios et al., 2008; Welsh et al., 2013). By contrast, our results indicate that stomach curvature is driven by asymmetric morphogenesis within the organ itself.

Moreover, whereas Pitx2c elicits tissue condensation in left dorsal mesentery (Davis et al., 2008), Pitx2c elicits tissue expansion in the left stomach, suggesting that the final manifestation of Pitx2c activity in different organs is not universal, but is influenced by...
geometric context (e.g. internal or external to the gut tube) and tissue type. Our study suggests that the final topology of the digestive tract is the end-product of at least two Pitx2-mediated symmetry-breaking processes: mesentery distortion, which positions the gut tube asymmetrically in the body cavity; and asymmetric gut morphogenesis, which sculpts disparate morphologies within the contralateral walls of the gut itself.

MATERIALS AND METHODS

Animals and genotyping

Animals used in accordance with NCSU IACUC regulations. Xenopus in vitro fertilization, embryo culture and staging were as described previously (Nieuwkoop and Faber, 1994; Sive et al., 1998). Heterozygous Foxj1+/creERT2 mice (Muthusamy et al., 2014) were used in timed pregnancy matings to obtain Foxj1+/creERT2 (i.e. Foxj1+/+) and Foxj1creERT2+/creERT2 (i.e. Foxj1+/−) embryos. Noon on day of vaginal plug discovery was defined as E0.5. Embryos were harvested in ice-cold 0.1 M phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde in PBS. PCR was used to identify homozygous creERT2 insertion into Foxj1 locus (Muthusamy et al., 2014); see supplementary Materials and Methods for genotyping primers.

Xenopus loss- and gain-of-function experiments

Pharmacological (e.g. SB505124) dosing of Xenopus neurulae (stage 19/20) was conducted as described previously (Dush et al., 2011). SB505124 was diluted to 5 μM in culture medium (0.1× MMR); an equal volume of DMSO (solvent) was used as a control. Microinjection was performed at 1-cell (CRISPR) or 8-cell stages (morpholinos and mRNA), using established methods (Sive et al., 1998). Co-injected GFP mRNA validated tissue targeting. Pitx2-GR mRNA-injected embryos were exposed to dexamethasone (10 µM) or to ethanol (solvent) as used as a control.

Morphometric measurements performed with ImageJ, Photoshop or eMouse measuring tools, EMAP eMouse Atlas Project (http://www.emouseatlas.org). Statistical significance was determined by ANOVA with post-hoc Tukey HSD; see supplementary Materials and Methods for details.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.D., N.M.A., C.J. and K.B. performed experiments; N.N.-Y. conceived of the project and wrote manuscript; H.T.G. provided mouse strains.

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